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# Experimental exposure of farmed mink (*Neovison vison*) to livestock-associated methicillin-resistant *Staphylococcus aureus* contaminated feed

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## ABSTRACT

Livestock-associated methicillin-resistant *Staphylococcus aureus* (LA-MRSA) is widely distributed in the Danish pig production. Spillover to the mink production is hypothesized to occur via contaminated pig by-products used in the production of mink feed.

The aim of the present longitudinal experimental cohort study was to confirm the potential of LA-MRSA being transmitted to naïve mink after exposure to contaminated feed, and to study the persistence of the bacterium on the animals after ceased exposure to contaminated feed.

LA-MRSA-negative mink ( $n = 28$ ) were housed in pairs in 14 mesh cages. Twenty-four mink (12 cages) received around  $5.1 \times 10^8$  cfu/mink in the feed for five days, while four mink (two cages) were kept as negative controls and fed with LA-MRSA negative feed. Twenty-four hours after initiation of spike, all 28 mink were tested LA-MRSA-positive by paw swabs. After cease of the spiking period, one mink per cage were moved to a clean housing facility to study the potential effect of environmental contamination in persistence of the LA-MRSA. All mink were re-tested three times per week for the subsequent 26 days to study whether the mink cleared off the bacterium.

The results showed that LA-MRSA can be transmitted to paws and pharynx on mink after exposure to contaminated feed and that LA-MRSA may spread indirectly through contaminated environmental sites. Mink tend to clear off LA-MRSA, however, the bacterium may persist on mink for more than 26 days.

## 1. Introduction

Livestock-associated methicillin-resistant *Staphylococcus aureus* (LA-MRSA) has been isolated from pharynx and paws of clinically healthy farmed mink (*Neovison vison*). On farms with LA-MRSA positive mink, LA-MRSA was found in 20% [13;29]<sub>CI95%</sub> to 29% [22;38]<sub>CI95%</sub> of the animals and distributed widely in the environment (Fertner et al., 2019). In contrast to pigs where LA-MRSA are primarily located in the nose and on skin, LA-MRSA in mink are primarily isolated from paws and pharynx (Hansen et al., 2017). Hence, LA-MRSA on mink farms poses a public health hazard to farmers if exposed to colonized animals through bites and scratches or contaminated environmental sites. In Denmark, 6000 people are employed in the mink industry constituting the world's largest producer of mink (Kopenhagen Fur, 2017, 2019).

In humans and other animal species, LA-MRSA is transmitted via direct contact and is not considered feedborne. However, in mink, there is good evidence that the bacterium is in fact introduced by feed. LA-MRSA in mink is believed to originate from contaminated pig by-

products used in the production of mink feed, due to three main reasons as presented by Hansen et al. (2017). Firstly, the primary anatomical site of isolation on the mink is the pharynx and paws, which are in close contact with feed particles. Secondly, the most prevalent LA-MRSA *spa*-types (t034 and t011) found in mink correspond to those dominating in Danish pig farms. Thirdly, 19% (20/108) of mink feed samples analyzed in 2016 were found positive for LA-MRSA (Hansen et al., 2017).

As feed is suspected to be the primary source of LA-MRSA colonization in mink, mink may clear themselves if the source of introduction is removed. The primary objective of the present study was to confirm whether LA-MRSA-contaminated feed could serve as a source for LA-MRSA transmission in mink; hence, to study whether LA-MRSA could be re-isolated from paws in mink fed with LA-MRSA spiked feed. Secondly, we wanted to study the persistence of LA-MRSA after the spiking period was ended and to evaluate the effect of housing mink in clean facilities.

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## 2. Materials and methods

### 2.1. Test facilities and animals

We performed a longitudinal cohort-study in 28 farmed brown mink (*Neovison vison*) bought in as 14 pairs (male and female) from one single supplier, and entered the test facilities on November 9, 2017. The mink were six months of age, which is fully grown mink and the typical age for pelting. The study was carried out in a thoroughly cleaned closed test facility intended for animal experiments. Each room held 14 connected standard mesh-cages separated by solid plates, identical to those used in commercial mink farms. Hence, the mink were in direct proximity to neighbors. Change of clothes and boots were required upon entrance to each test room. Both isolated test-rooms were held at a constant temperature of 15 °C and an air humidity of 75%.

Prior to the spike, all mink had both their forepaws swabbed to assure LA-MRSA negative status. The mink were fed commercial mink feed that prior to feeding was heat treated to 60 °C kernel temperature and tested to confirm LA-MRSA negative status.

Initially, all mink were housed in the pairs of one female and one male. At day one to five, four mink (cages no 1-2) were kept as negative controls, while 24 min. (cages no 3-14) were fed commercial feed supplemented with an LA-MRSA culture,  $3.4 \times 10^6$  cfu/g. Immediately after the spiking period, half the mink were relocated into an identical clean test room, as one mink from each cage was moved, alternate male-female. Hereafter all mink were housed single in cages; 14 min. in the contaminated environment where the spiking took place and 14 min. in a clean environment (Fig. 1). All mink had both forepaws swabbed three times per week during the study period. The swab samples were taken after fixation of the animal using moistened, sterile swab cloths (SodiBox Swab Cloth, SodiBox, Nevez, France). The samples were collected manually by gently rubbing all the surfaces of the paw with the cloth. Gloves were worn and changed between animals. The first sample was taken 24 h after the first spike. If one mink was tested negative on three consecutive samplings, the mink was perceived as negative and was euthanized in order to minimize the stress of handling. The remaining mink were all euthanized at day 31 in the study period, 26 days after the last feeding with spiked feed. From each carcass, a pharyngeal swab was taken and the right forepaw dissected at the carpal joint. Both samples were stored in clean plastic containers

and transported to the laboratory for selective culture of LA-MRSA (Fig. 1).

### 2.2. Bacterial culture

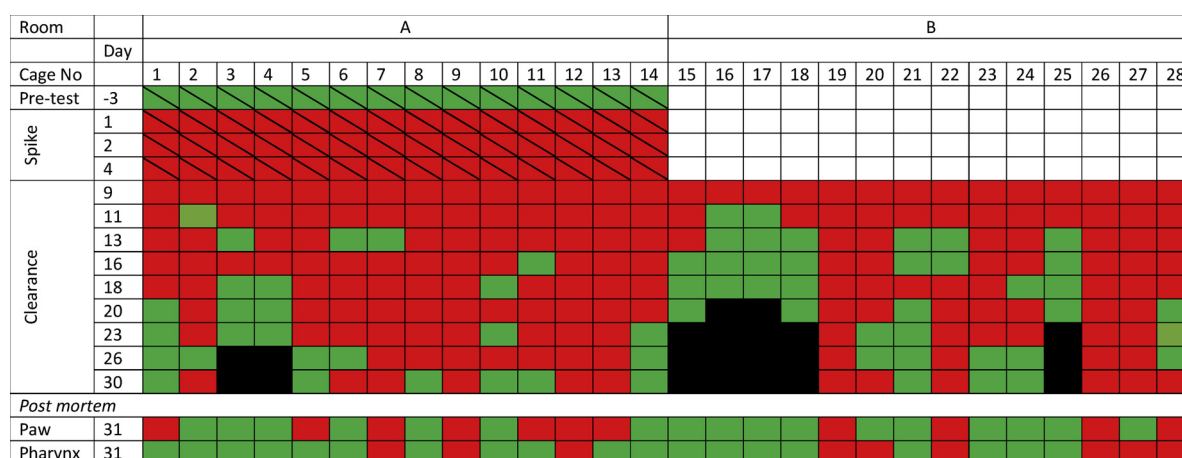
For the spike culture, we used a typical LA-MRSA CC398 which previously had been isolated from the pharynx of a mink (Fertner et al., 2019), belonging to the L3, which is one of the three major lineages found in the Danish pig production (Sieber et al., 2018). A loop-full of cultured bacteria was mixed into 1 l of Mueller-Hinton broth with 6.5% NaCl (Oxoid, Basingstoke, United Kingdom) and incubated at 37 °C for 24 h, reaching a concentration of  $3.4 \times 10^8$  cfu/ml. Subsequently, the culture was stored at 5 °C at the test facility. During the spiking period, the culture was mixed into the feed on a daily basis; 1 ml of cultured broth per 100 g feed, yielding a final concentration of around  $3.4 \times 10^6$  cfu/g feed. Each cage of two mink received 300 g feed, corresponding to an average amount of ingested LA-MRSA of  $5.1 \times 10^8$  cfu/mink. It was controlled that the complete amount of feed was ingested by the pair.

Müller-Hinton broth with 6.5% NaCl (100 ml) was added to the SodiBox swab cloths in the original plastic bags and stomached for 30 s at 230 rpm (Stomacher 400 circulator, Seward). Likewise, pharyngeal swabs and dissected paws in separate clean plastic containers were respectively added 3 ml and 30 ml Müller-Hinton broth with 6.5% NaCl. Hereafter, all samples were incubated at 37 °C for 18–24 h. From the overnight culture, 10 µl were streaked onto Brilliance™ MRSA2 agar plates (Oxoid) and incubated at 37 °C for 18–24 h where after suspect colonies were assessed using matrix-assisted laser desorption ionization (MALDI-TOF mass spectrometry).

### 2.3. Data handling

Throughout the study period, results from the bacterial culture were continuously entered manually into Excel 2016 from the Microsoft Office Package. After completion of the results, the final dataset was exported to R version 3.4.1 for data handling (R Core Team, 2014).

Due to few observations ( $\leq 6$ ) in one or more categories, a Fisher's exact test was used to test for differences in prevalence of LA-MRSA between sexes and between test rooms (clean and contaminated facilities), for both paw and pharyngeal swabs. Further, a McNemar's chi squared test was used to test the difference in prevalence of LA-MRSA



**Fig. 1. Results from a longitudinal study in mink spiked with LA-MRSA in the feed and followed the proceeding 26 days.** During the first seven days, mink were housed in pairs (male and female). Three days prior to the feed spike, all mink were pre-tested to assure LA-MRSA-negative status. On day one to five, 24 of the mink (cage number 3-14) were spiked with around  $5.1 \times 10^8$  cfu LA-MRSA/mink, while four mink (cage no 1-2) were kept as negative controls. Hereafter, one mink (altering male and female) from each cage was moved to a clean test room. Negative control mink from cage no 1 and 2 were moved to cage no 15 and 16 in the clean room. From this step on, all mink were housed in separate cages. During the whole study period, all mink were re-tested by paw swabs to test for LA-MRSA three times per week. At the end of the study period, the right forepaw and pharyngeal swabs were cultured for LA-MRSA on all mink *post mortem*. Colours indicate the status of LA-MRSA tested. Green: LA-MRSA negative samples. Red: LA-MRSA positive samples. Black: Mink have been euthanized (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

between the paired samples of paw and pharyngeal swabs.

### 3. Results

A timeline of the study, including rate of clearance of LA-MRSA in the 28 min., is presented in Fig. 1. No clinical symptoms in the mink were observed during the study period.

*Post mortem* findings showed a significant difference ( $p$ -value = 0.02) in the proportion of LA-MRSA positive paw samples between males (9/14) and females (2/14), while no significant difference ( $p$ -value = 0.42) was observed in the proportion of LA-MRSA positive pharyngeal swabs between males (6/14) and females (3/14).

With regard to difference in LA-MRSA positive samples at the end of the study period between mink housed in the contaminated room (paw 7/14, pharynx 3/11) and the clean room (paw 4/14, pharynx 6/14), we found no significant difference in LA-MRSA-prevalence for neither paw samples ( $p$ -value = 0.44) nor pharyngeal swabs ( $p$ -value = 0.42). Likewise, for paw samples and pharyngeal samples (McNemar's chi-squared test = 0.17,  $p$ -value = 0.68). This means that no significant difference in prevalence between neither rooms (clean/contaminated) nor anatomical site (paw/pharynx) was found.

### 4. Discussion

Results from the present study confirmed the hypothesis, that LA-MRSA could be re-isolated from mink paws and pharynx after exposing mink to contaminated feed. Paw swabs from both spiked mink (24) and non-spiked mink (4) were found LA-MRSA positive within 24 h after exposure to the contaminated feed, indicating that LA-MRSA may disperse in the environment and has the potential to spread to mink held in neighboring mesh-cages. None of the four negative control mink had LA-MRSA isolated from pharynx, suggesting that they had attracted the bacterium via direct contact with colonized animals or contaminated surfaces and not by ingestion of contaminated material. Direct animal-animal and indirect animal-environment-animal transmission routes have also been demonstrated for LA-MRSA CC5 in pigs (Gibbons et al., 2013), where LA-MRSA was re-isolated from nares in 66–75% of the pigs immediately after environmental exposure.

The 100% colonization rate in the present study may be due to the relatively high amounts of LA-MRSA exposed to each mink, namely  $5.1 \times 10^8$  cfu/animal. Quantitative studies on LA-MRSA are sparse. Hansen (2017) quantified the load of LA-MRSA on the surface of gilts, and found a median of 30 cfu/swab, range 5–690 cfu/swab, from swabs behind the ear pinna. Concentrations of LA-MRSA in mink feed are expected to be substantially smaller. Firstly, we did not know what the minimum dose for colonization would be, and secondly, we aimed at a high colonization rate enabling to study the persistence of LA-MRSA on mink. Therefore, we chose a high spiking dose, well aware that this dose does not reflect conditions in the field. A future study on the quantification of LA-MRSA in commercial mink feed along with a study of effective dose for colonization in mink would be highly relevant. This would enable the establishment of a minimal colonization dose, IC<sub>50</sub>.

A significant higher proportion of LA-MRSA positive paw samples was observed for male mink compared to female mink, possibly explained by a higher feed consumption or a larger surface area of the paws. No difference was observed in the proportion of LA-MRSA positive pharyngeal swabs.

Paws are expectedly more influenced by environmental contamination than the pharynx. At the end of the study period, almost twice as many paws were found positive *post mortem* in the contaminated room (7/14), compared to the clean room (4/14). Yet, the difference was not found significant, possibly due to a too small sample size.

Following the spiking period, LA-MRSA-positive mink tended to

gradually eliminate the bacterium. However, at the end of the study, LA-MRSA could still be re-isolated from 11/28 of the mink, meaning that LA-MRSA is able to persist on mink for more than 26 days after removal of the contaminated feed. Currently, we do not know whether mink may have to potential become colonized with LA-MRSA or merely serve as carriers for the bacterium. Studies on potential multiplication of the bacterium on mink would therefore be relevant. Nevertheless, despite the relatively high bacterial dose of LA-MRSA used in the spiking culture of the current study, none of the 28 min. developed clinical infections during the study.

LA-MRSA has in another study been shown to distribute widely in the environment of LA-MRSA positive mink farms (Fertner et al., 2019). Hence, removal of the contaminating feed source along with a proper cleaning is needed to eliminate LA-MRSA from the facilities.

### 5. Conclusion

In the present study, we confirmed the exposure to contaminated feed as a likely route of LA-MRSA colonization of mink. All exposed mink became LA-MRSA positive on paws 24 h after feed spike. The majority of mink tend to clean off the bacterium after removal of the oral exposure, although the potential of mink being persistently colonized with LA-MRSA cannot be rejected. Further, LA-MRSA may spread indirectly in the environment to neighboring mink.

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### Conflict of interest

None to declare.

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